Location of the Calcium Ion Binding Site in Porcine Pancreatic Elastase Using a Lanthanide Ion Probe

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ABSTRACT: Calcium and gadolinium ions are able to bind to elastase at identical sites and with similar affinities. The dissociation constants of the elastase calcium and gadolinium complexes (4.5×10^{-5} and 2×10^{-5} M, respectively, at pH 5 and 35 °C) are not significantly affected by the presence of a specific inhibitor of this enzyme, trifluoroacetyltrialanine. Conversely, the binding of calcium to elastase does not modify the enzyme's affinity for the inhibitor, nor its reactivity toward its specific substrate, succinyltrialanine-*p*-nitroanilide. The relaxation times T_{1EIM} and T_{2EIM} of the fluorine nuclei of the trifluoroacetyltrialanine in the ternary elastase-inhibitor-gadolinium ion complex have been obtained by factorization of the observed relaxation times in terms of exchange and true relaxation contributions. The distance from the gadolinium ion to the trifluoroacetyl group of the inhibitor has then been calculated to be 20 Å by using the Solomon-Bloembergen equations. This rather large distance explains in part the independence of the two fixation sites of the ion and the inhibitor. The previous demonstration that the trifluoroacetyl group binds in the vicinity of one of the S sites, together with crystallographic coordinates of pancreatic elastase, permits us to conclude that the fixation site of the calcium ion to elastase in solution is most probably the same as that of a uranyl ion to this enzyme in the crystal, i.e. the carboxylic side chains of Glu-70 and Glu-80 (based on the chymotrypsin sequence numbering system). In addition, the rate constant k_{off} of dissociation of the elastase-peptide complex, measured under a large concentration range, is almost constant, thus suggesting that the trifluoroacetyltrialanine is bound in a unique mode to the enzyme.

Calcium ion binding on serine proteases involves specific sites of the proteins. One calcium ion is bound to bovine trypsin in the crystal at a site which includes the Glu-70[1] and the Glu-80 side chains (Bode and Schwager, 1975). This site is different, however, from that assigned for the binding of a calcium ion to bovine trypsin (Abbott et al., 1975; Darnall et al., 1975) and chymotrypsin A (Birnbaum et al., 1977) in solution.

These authors conclude from fluorescence energy transfer and nuclear magnetic resonance (NMR)[2] experiments using lanthanide probes that the calcium ion binding site in solution is on the side chains of Asp-194 and Ser-190. They reported also that pancreatic elastase has a very low affinity for a calcium ion, probably due to the lack of the Ser-190 hydroxyl group in this enzyme (Darnall et al., 1975). The Glu-70 and Glu-80 residues, however, are still present in this protease and they bind to a uranyl ion in the crystal (Shotton and Watson, 1970).

As will be shown in this paper, the above conclusion of Darnall et al. on elastase is not correct: calcium, as well as gadolinium ions, form stable complexes with elastase. Moreover, the lanthanide ion may be used as a paramagnetic probe for exploring the vicinity of the ion binding site at the surface of the protein. We used for this latter purpose the NMR of the fluorine label of a trifluoroacetylated trialanine peptide, which has been previously shown (Dimicoli et al., 1976) to form a stable complex with elastase.

Materials and Methods

Elastase and Trifluoroacetyltrialanine. Porcine pancreatic elastase and trifluoroacetyltrialanine were prepared and assayed as previously reported (Bieth et al., 1974; Dimicoli et al., 1976). The various kinetics and inhibition parameters and their 95% confidence intervals were obtained using a program of nonlinear regression.

Enzyme-Ion Binding Equilibria. Enzyme-ion binding was measured by equilibrium dialysis under the following conditions: 2×10^{-4} M protein, 5×10^{-2} M acetate buffer at pH 5, or 0.1 M Tris buffer at pH 8, and various concentrations of NaCl. Such conditions prevent the Donnan effect from effecting the relative ion concentrations in both compartments of the cell. On the other hand, it can be easily shown that the effect of complex formation of lanthanides with acetate ion (Sonesson, 1958), in large and constant concentration in the medium, is limited to the competition of the acetate ion with the enzyme, leading to a lower apparent affinity of the enzyme for the lanthanide ion. In addition, approximately 10^{-8} M $^{45}CaCl_2$ (IRE, Fleurus, Belgium) was present in each dialysis cell. After equilibration at 35 or 5 °C during a 15-18-h period, the emissions from the two compartments of each cell were monitored using a Intertechnique SL32 scintillation counter. The difference between the numbers of counts emitted by equal volumes of the compartments containing and not containing the enzyme, C_1 and C_2, respectively, proved the existence of binding between the $^{45}Ca^{2+}$ ion to the enzyme. Moreover, as the concentration of the $^{45}Ca^{2+}$ ion is much lower than that of the free enzyme, this latter concentration, E, can be obtained from the relation:

$$E = \frac{K_O(C_1 - C_2)}{C_2} \qquad (1a)$$

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[1] Throughout this paper, we shall use both the chymotryptic numbering system for the amino acid sequence of serine proteases (Stroud et al., 1971) and the subsite nomenclature of Schechter and Berger (1967) for the active site of proteases.

[2] Abbreviations used are: NMR, nuclear magnetic resonance; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

where K_O is the overall dissociation constant of the elastase $^{45}Ca^{2+}$ ion complex, and is obtained in the absence of any other added ion. For this latter condition, the relation 1a is still valid, with the total enzyme concentration, e_0, appearing in the place of the concentration E.

On the other hand, the total quantity of the nonradioactive ions (Ca^{2+} or Gd^{3+}) was determined at the beginning of the experiments by weighing the $CaCl_2$ and by complexometric titrations by EDTA using xylenol orange (Lyle and Rahman, 1963) as an indicator in 0.2 M acetate buffer (pH 5.6), for gadolinium nitrate·$5H_2O$.

The free enzyme concentration, determined by counting the $^{45}Ca^{2+}$ ion containing solutions, and the total nonradioactive ion concentration permit us to evaluate the free, and enzyme-bound, nonradioactive ion concentrations. A Scatchard plot (Scatchard, 1949) can then be constructed that gives an initial estimation of the number of sites of fixation of each ion on the enzyme and the corresponding affinity constant. For a unique site of fixation which was always the case in the present experiment, the affinity constant could be refined and its 95% confidence interval obtained by a nonlinear regression analysis of the experimental data using the expression giving the concentration of the enzyme-bound ion, EM:

$$EM = \frac{E_0 + M_0 + K - [(E_0 + M_0 + K)^2 - 4E_0M_0]^{1/2}}{2} \quad (1b)$$

where M_0 and K are the total ion concentration and the dissociation constant of the enzyme–ion complex, respectively.

NMR. The NMR spectra were recorded at 100 (^1H) and 94 MHz (^{19}F) using a Varian XL100 spectrometer operating in the Fourier transform at a sample temperature of 34 °C. The chemical shifts were referenced to the resonance of trifluoroacetic acid in a coaxial capillary.

The elastase solutions in D_2O–5×10^{-2} M acetate buffer were prepared without NaCl, ensuring complete solubility of the enzyme up to 10^{-3} M. Spin–lattice relaxation times, T_1, were measured by the induction recovery method (Pratt and Sykes, 1972), using 180 °C–τ–90 °C–t sequences. Spin–spin relaxation times, T_2, were measured from the half-height line width of the resonance line ($T_2 = 1/\pi\Delta\nu$), using an appropriate spectral resolution. No oxygen effects requiring systematic degassing of the solutions were observed for these measurements.

In enzyme-inhibitor systems in which the chemical exchange is fast with respect to the difference in chemical shift for the observed nucleus of the inhibitor free in solution (I) and complexed to the protein (EI), i.e. when a single line is observed, the relaxation rates are given by the following relations (Smallcombe et al., 1972):

$$\frac{1}{T_1} = \frac{p_I}{T_{1I}} + \frac{p_{EI}}{T_{1EI}} \quad (2a)$$

$$\frac{1}{T_2} = \frac{p_I}{T_{2I}} + \frac{p_{EI}}{T_{2EI}} + \frac{p_I^2 p_{EI}(\omega_{EI} - \omega_I)^2}{k_{off}} \quad (3a)$$

In these relations, T_i (i, 1, 2) is the observed relaxation time, T_{iI} and T_{iEI} are those for the free and complexed inhibitor, respectively, and p_I, p_{EI}, ω_I, and ω_{EI} are the relative proportions and frequencies of the nuclear precession in the two forms, respectively. It should be noted that the last term in eq 3a, which represents the contribution of the chemical exchange to the line width, is maximum for $p_I = 2/3$. The dissociation rate constant of the complex, k_{off}, may be derived from these relations once p_I and p_{EI} have been determined from the measurements of the chemical shift of the observed resonance:

$$\delta = p_I\delta_I + p_{EI}\delta_{EI} \quad (4a)$$

To obtain p_I and p_{EI} from relation 4a, a trial starting value of δ_{EI} is chosen that allows a first estimation of the number of fixation sites for the binding of the inhibitor to the enzyme, and of the corresponding dissociation constants, using a Scatchard representation. In the case of a unique site of fixation, these first estimations of the chemical shift, δ_{EI}, and of the dissociation constant, K_d, can be refined and their 95% intervals obtained by a nonlinear regression analysis of the experimental data using the expression giving the observed chemical shift:

$$(\delta - \delta_I) = \frac{E_0 + I_0 + K_d - [(E_0 + I_0 + K_d)^2 - 4E_0I_0]^{1/2}}{2I_0} \times (\delta_{EI} - \delta_I) \quad (5a)$$

where I_0 is the total concentration of the inhibitor.

The ratio k_{off}/K_d gives, finally, the second-order rate constant k_{on} of formation of the enzyme-inhibitor complex.

The analytical expression for the relaxation times T_1 and T_2 of a nucleus in an inhibitor able to exchange chemically between three sites, the free inhibitor (I), the enzyme-inhibitor complex (EI), and the ternary complex with a metal ion (EIM), can be obtained through calculations similar to those used by Swift and Connick (1962) and Reuben and Fiat (1969). Under the following experimentally observed conditions—no change of chemical shifts upon addition of metal ($\delta_{EI} = \delta_{EIM}$), no change in the dissociation and association rate constants, k_{off} and k_{on}, for the enzyme-inhibitor complex upon addition of metal ion, fast chemical exchange rates relative to the relaxation times—relations 2b, 3b, and 4b hold:

$$\frac{1}{T_1} = \frac{p_I}{T_{1I(M)}} + \frac{p_{EI}}{T_{1EI}} + \frac{p_{EIM}}{T_{1EIM}} \quad (2b)$$

$$\frac{1}{T_2} = \frac{p_I}{T_{2I(M)}} + \frac{p_{EI}}{T_{2EI}} + \frac{p_{EIM}}{T_{2EIM}} + \frac{p_I^2(p_{EI} + p_{EIM})(\delta_{EI} - \delta_I)^2}{k_{off}} \quad (3b)$$

$$\delta = p_I\delta_I + (p_{EI} + p_{EIM})\delta_{EI} \quad (4b)$$

in which the index EIM refers to the ternary enzyme-inhibitor-metal complex, and $T_{iI(M)}$ is the relaxation time of the peptide in the presence of enzyme and ion.

Equation 3b may be rewritten in a condensed form:

$$\frac{1}{T_2'} = \frac{1}{T_2} - \frac{1}{T_{2,exch}} \quad (3c)$$

where $1/T_{2,exch}$ is the contribution of chemical exchange to $1/T_2$ corresponding to the last term of eq 3b, which is the same as in the diamagnetic case (eq 3a) and $1/T_2'$ includes only the contributions of the enzyme and paramagnetic ion to the relaxation.

The extrapolated values of $1/T_1$ and $1/T_2'$ for $P_{EIM} = 1$ allow us to estimate T_{1EIM} and T_{2EIM} and to derive the values of τ_c and r, by using the following simplified Solomon–Bloembergen equations ignoring the contributions due to the modulation of the scalar interaction between the electron spins on the gadolinium ion and the nuclear spins:

$$1/T_{1EIM} = \frac{2S(S+1)}{15r^6}(g\mu_B\gamma_I)^2 \times \left[\frac{3\tau_c}{1 + \omega_I^2\tau_c^2} + \frac{7\tau_c}{1 + \omega_S^2\tau_c^2}\right] \quad (5b)$$

TABLE I: The Dissociation Constants, K, of the Complexes of Ca^{2+} with Porcine Pancreatic Elastase and Other Serine Proteases.

Enzyme	pH	Temp (°C)	Ionic strength	Effector	K (M)	Ref
Porcine elastase	5	35	0.2	0	$1.4 \pm 0.3 \times 10^{-4}$	*a*
	5	35	0.05	0	$4.5 \pm 0.5 \times 10^{-5}$	*a*
	5	35	0.05	Trifluoroacetyltrialanine	$4.3 \pm 0.5 \times 10^{-5}$	*a*
	8	35	0.2	Trifluoroacetyltrialanine	$2 \pm 0.7 \times 10^{-5}$	*a*
	8	4	0.2	0	$7.3 \pm 0.6 \times 10^{-5}$	*a*
Bovine trypsin	6	25	0.3	*p*-Toluamidine-chloride	3.8×10^{-3}	*b*
	8	25	0.3	*p*-Toluamidine-chloride	3.0×10^{-4}	*b*
Porcine trypsin	6.3	5	0.2	0	1.6×10^{-4}	*c*

[a] This work. [b] Abbott et al. (1975). [c] Epstein et al. (1974).

TABLE II: The Dissociation Constants, K, of the Complexes of Gd^{3+} with Porcine Pancreatic Elastase and Other Serine Proteases.

Enzyme	pH	Temp (°C)	Ionic strength	Effector	K_d (M)	Ref
Porcine elastase	5	35	0.15	0	$8.5 \pm 2 \times 10^{-5}$	*a*
Porcine elastase	5	35	0.05	0	$3.3 \pm 1 \times 10^{-5}$	*a*
Porcine elastase	5	35	0.05	Trifluoroacetyltrialanine	$2 \pm 0.6 \times 10^{-5}$	*a*
Bovine trypsin	6	25	0.3	*p*-Toluamidine-chloride	3×10^{-4}	*b*
Bovine chymotrypsin A	6	25	0.3	*p*-Toluamidine-chloride	5×10^{-4}	*d*
Porcine trypsin	6.3	5	0.2	0	2.9×10^{-3}	*c*

[a] This work. [b] Abbott et al. (1975). [c] Epstein et al. (1974). [d] Birnbaum et al. (1977).

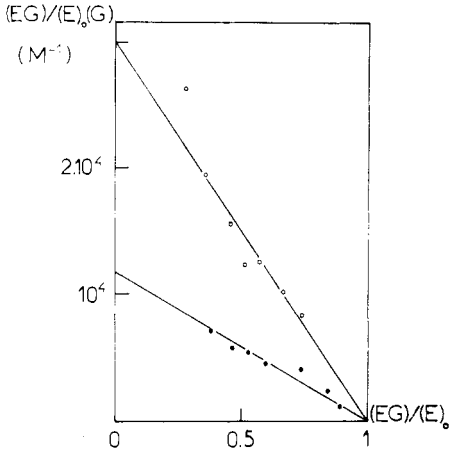

FIGURE 1: Scatchard plot for the interaction of elastase with gadolinium ion in 5×10^{-2} M acetate buffer (pH 5) with (●) and without (○) 10^{-1} M NaCl at 35 °C, as obtained by competition with $^{45}Ca^{2+}$ ion.

$$1/T_{2EIM} = \frac{S(S+1)}{15r^6}(g\mu_B\gamma_I)^2 \times \left[4\tau_c + \frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{13\tau_c}{1+\omega_S^2\tau_c^2}\right] \quad (5c)$$

where S is the electron spin quantum number for gadolinium ($^7/_2$), γ_I is the fluorine magnetogyric ratio (2.518×10^4 rad/(s G)), g is the electronic "g" factor (2), μ_B is the Bohr magneton (0.92731×10^{-20} erg/G), ω_I is the Larmor angular precession frequency for nuclear spin of fluorine (5.91×10^{10} rad/s), and ω_S is the Larmor angular precession frequency for electron spins (4.15×10^{11} rad/s).

Results

(1) *Binding of Ca^{2+} and Gd^{3+} Ions to Elastase.* The dissociation constants of the $^{45}Ca^{2+}$ elastase complex, measured by equilibrium dialysis under various conditions of pH, temperature, and ionic strength, and in the presence or absence of the trifluoroacetylated inhibitor, are reported in Table I. They are comparable to those measured for other serine proteases, suggesting the presence of a specific site for this ion. Competition experiments with nonradioactive calcium ions confirm the uniqueness of this site. In addition, the presence of the inhibitor has no effect on calcium binding.

The binding of trivalent gadolinium ion to elastase is also competitive with that of $^{45}Ca^{2+}$ ion (Figure 1). The affinity of elastase for this metal ion has been confirmed by direct titration of Gd^{3+} ion (Table II). It is not affected by the presence of the elastase inhibitor and its value which is a minimal one due to the competition effect of acetate ion present in large excess (see above) is comparable or even higher than that for other serine proteases.

(2) *Effect of Ca^{2+} and Gd^{3+} Ions on the Properties of Elastase.* Table III shows that Ca^{2+} and Gd^{3+} ions affect neither the kinetic parameters of the elastase-catalyzed hydrolysis of succinyltrialanine-*p*-nitroanilide, nor the dissociation constant of the elastase-trifluoroacetyltrialanine complex. These results are in agreement with the data of Tables I and II and demonstrate that the metal ion binding site and the substrate (inhibitor) binding site are quite different and independent.

The rate of autolysis of the enzyme (ca. 20% after 15 h at 35 °C and pH 5) is slowed down by the trifluoroacetyltrialanine inhibitor, but it is not further affected by the metal ions, thus confirming that the fixation of these ions and of the inhibitor is not mutually exclusive.

(3) *NMR Investigation of the Elastase–Trifluoroacetyltrialanine Interactions.* The ^{19}F resonance of this specific inhibitor of elastase has already been shown (Dimicoli et al., 1976) to be strongly dependent upon the presence of the enzyme. A quantitative analysis of its behavior in the ternary complex obtained with gadolinium ions first requires the estimation of the contribution of chemical exchange in the binary enzyme–inhibitor complex. The titration of the enzyme by the inhibitor results in a high field of the fluorine resonance with broadening, then narrowing, of the corresponding line. The maximum line width is observed for a p_I value around $^2/_3$ (Figure 2a), indicating the importance of chemical exchange in this process. The observed chemical-shift variations allow

TABLE III: The Effect of the Calcium and Gadolinium Ions upon the Hydrolysis by Porcine Pancreatic Elastase (k_{cat}, K_m) of Succinyltrialanine-*p*-Nitroanilide, and Its Inhibition (K_I) by Trifluoroacetyltrialanine at 35 °C and Various Conditions of pH and Ionic Strength.

Effector	Reaction	pH	Ionic strength	Temp (°C)	Ion	K_m (M)	k_{cat} (s^{-1})	K_I (M)
Succinyltrialanine-*p*-nitroanilide	Hydrolysis	5	0.05	35	0	$2.4 \pm 0.3 \times 10^{-3}$	3 ± 0.3	
		5	0.05	35	Gd^{3+} (8×10^{-4} M)	$2.3 \pm 0.3 \times 10^{-3}$	3 ± 0.3	
Trifluoroacetyltrialanine	Inhibition	5	0.05	35	0			$5.2 \pm 0.5 \times 10^{-5}$
		5	0.05	35	Gd^{3+} (8×10^{-4} M)			$4 \pm 0.5 \times 10^{-5}$
Succinyltrialanine-*p*-nitroanilide	Hydrolysis	8	0.2	25	0	$1.2 \pm 0.3 \times 10^{-3}$	17 ± 2	
		8	0.2	25	Ca^{2+} (10^{-3} M)	$1 \pm 0.3 \times 10^{-3}$	18 ± 3	

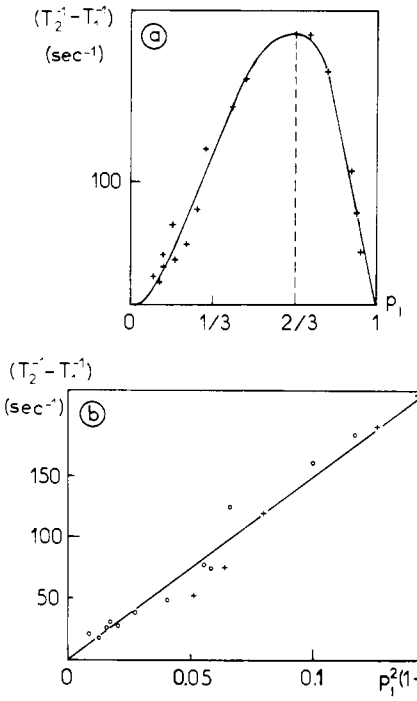

FIGURE 2: (a) Relaxation rate ($T_2^{-1} - T_1^{-1}$) due to the chemical exchange for the fluorine resonance of trifluoroacetyltrialanine as a function of p_I, the fraction of inhibitor in the free state in the presence of 1.3×10^{-3} M elastase in 5×10^{-2} M acetate buffer, pD 5, 35 °C. (b) Plot of ($T_2^{-1} - T_1^{-1}$) (cf. a) vs. $p_I^2(1 - p_I)$ at pD 5, in 5×10^{-2} M acetate buffer, 35 °C. The O and + points correspond to values of p_I respectively lower and higher than 2/3.

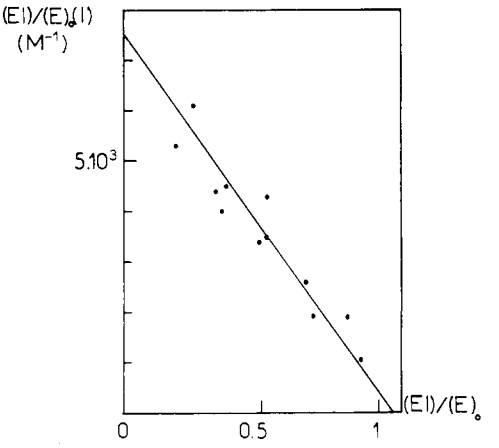

FIGURE 3: Scatchard plot for the interaction of elastase with trifluoroacetyltrialanine in 5×10^{-2} M acetate buffer, pD 5, 35 °C, as measured from the variations of chemical shift of the fluorine resonance of this peptide in various conditions of concentrations of enzyme and inhibitor.

us to initially obtain a Scatchard plot (Figure 3) showing that there is a single peptide inhibitor bound to the enzyme in this concentration range. The final value of the dissociation constant as obtained by nonlinear regression analysis, $K_d = 1.3 \pm 0.4 \times 10^{-4}$ M, is in good agreement with that measured by means of enzyme inhibition ($K_d = 5 \times 10^{-5}$ M) using much lower enzyme concentrations (10^{-8} M). The same regression analysis gives a fluorine chemical-shift variation in the complex of 149 ± 6 Hz at low-field values. Then p_I and p_{EI} can be more precisely calculated for each measurement. It can then be seen that there is a linear variation of ($T_2^{-1} - T_1^{-1}$) as a function of $p_I^2(1 - p_I)$ (Figure 2b), which would be expected from eq 2a and 3a when T_{1EI} and T_{2EI} are not significantly different. A value for k_{off} of 590 s^{-1} and of 4.4×10^6 M^{-1} s^{-1} for k_{on} has been derived from these relations. The times T_{1EI} and T_{2EI} are equal to 0.24 s. As already noted by Baldo et al. (1975), the value of k_{on} measured by NMR is far from that expected for a process controlled by diffusion and does not eliminate the possibility of stepwise binding, although the process is correctly described in the whole concentration range using a single value for the rate constants.

(4) *NMR in the Ternary Elastase-Gadolinium(III)-Peptide Complex.* When a solution of the trifluoroacetylated peptide is titrated by gadolinium ion in the absence of the enzyme, the fluorine relaxation times T_1 and T_2 are shortened progressively. The ratio of their variations is constant and approximately equal to 7/6. Such a value corresponds to a correlation time τ_c for the system dominated by the tumbling rate (Dwek, 1973) ($\tau_c \ll 10^{-9}$ s). The decrease of the relaxation time is much faster, especially for T_2, when elastase is present (Figure 4), but there is no significant difference in the frequency of the resonance line. There are, therefore, no significant differences in the parameters, including the induced chemical shift, describing the enzyme-inhibitor interaction when a ternary complex is formed. This was already noted during the equilibrium dialysis investigations. The relaxation rate, $1/T_2$, is still maximum for $p_I = 2/3$ and the line width variation must still be dominated by exchange phenomena. The extrapolation of the relaxation rate curves to $p_I = 0$ (Figure 5), i.e. in conditions where only $1/T_{iEI}$ and $1/T_{iEIM}$ contribute to the observed rates, shows that $1/T_{1EIM}$ and $1/T_{2EIM}$ cannot exceed 15 and 100 s^{-1}, respectively. The corresponding relaxation times are thus much longer than the lifetimes of the various species in solution which can be estimated from the kinetic rates derived in the previous paragraph. The relaxation behavior of the ternary system can thus be described by eq 2b to 4b. Using the value of k_{off} derived for the binary complex, the value of $1/T_2'$ (eq 3c) can be obtained. This value varies linearly with p_I since the p_{EI}/p_{EIM} ratio is constant at fixed

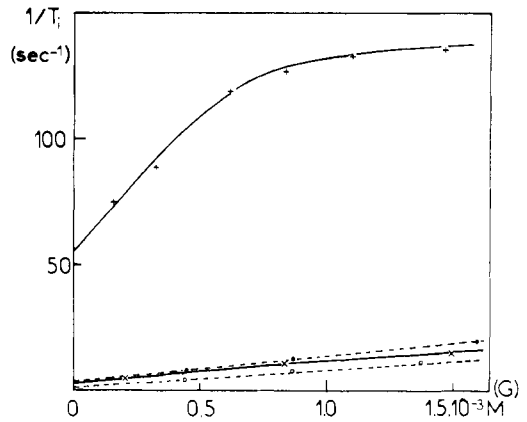

FIGURE 4: Effect of gadolinium ion concentration on the relaxation rates T_1^{-1} (O,×) and T_2^{-1} (●,+) of the fluorine nuclei of trifluoroacetyltrialanine (1.2×10^{-3} M), in the absence (- - -) and in the presence (—) of 1.2×10^{-3} M elastase in 5×10^{-2} M acetate buffer, pD 5, 35 °C.

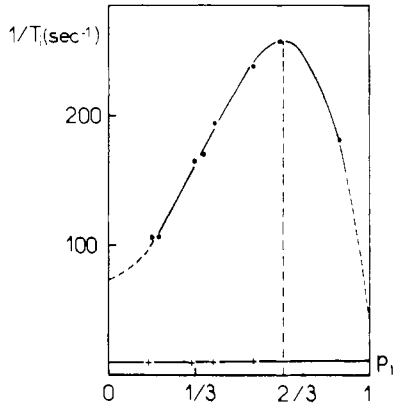

FIGURE 5: Relaxation rates T_1^{-1} (+) and T_2^{-1} (O) of the fluorine resonance of trifluoroacetyltrialanine in the presence of 1.1×10^{-3} M elastase and 7.9×10^{-4} M Gd^{3+} ion as a function of p_I, the fraction of inhibitor in the free state, in 5×10^{-2} M acetate buffer, pD 5, 35 °C.

gadolinium ion concentration (Figure 6). The extrapolation to $p_I = 0$ and 1 of the curves obtained for the values of $1/T_1$ and $1/T_2'$ as a function of p_I leads to the estimation of $1/T_{1EIM}$ ($15 \pm 1\ s^{-1}$), $1/T_{2EIM}$ ($100 \pm 20\ s^{-1}$), $1/T_{1I(M)}$ ($12 \pm 1\ s^{-1}$), and $1/T_{2I(M)}$ ($15 \pm 10\ s^{-1}$), respectively. The larger error of the T_2 values is due to the number of the intermediate steps in the calculations.

The ratio of the relaxation times of the fluorine nuclei of the inhibitor not bound to the enzyme in the presence of ternary complex, $T_{1I(M)}/T_{2I(M)}$, is still near unity. In the presence of a gadolinium ion, T_1 is nearly constant for both the free and enzyme-bound inhibitor ($T_{1I(M)} \simeq T_{1EIM}$). Since the tumbling rate is much larger in the former, the distance between the fluorine label and the gadolinium ion should increase markedly when the inhibitor is bound to the protein. In fact, the T_1/T_2 ratio of 7 observed for the ternary complex corresponds to a correlation time of 4.7×10^{-9} s and to a fluorine gadolinium ion distance of 20 Å. The precision on this estimate is still better than 10% in spite of the indirect method of calculation, due to the r^{-6} dependence of the magnetic dipole interaction.

Furthermore, this estimation of r excludes any significant nonspecific binding of the probe at a closer distance to the inhibitor as was observed in the case of trypsin (Epstein and Reuben, 1977). In our case such an eventual nonspecific binding, though not clearly demonstrated, would only lead to an underestimation of the distance r for which 20 Å is then a minimal value.

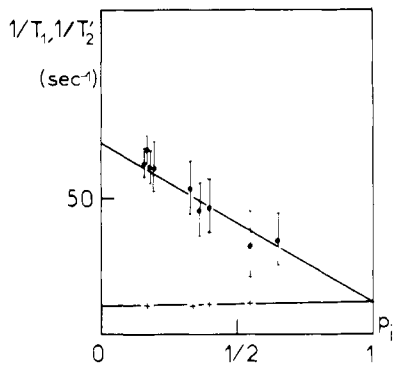

FIGURE 6: Plot of T_1^{-1} (+) and $T_2'^{-1}$ (●) (see the definition in the text) for the fluorine resonance of trifluoroacetyltrialanine, as a function of p_I, the fraction of inhibitor in the free state, in the presence of 1.1×10^{-3} M elastase and 7.9×10^{-4} M Gd^{3+} ion, in 5×10^{-2} M acetate buffer, pD 5, 35 °C. In such conditions, the ratio $p_{EIM}/(p_{EI} + p_{EIM})$ is equal to 0.7.

Discussion

The presence of a unique metal binding site in porcine pancreatic elastase has been firmly established by equilibrium dialysis and NMR spectroscopy. The finding disagrees with the results of Darnall et al. (1975), but is in accord with the binding of trivalent terbium ion to elastase, demonstrated by Brittain et al. (1976) while the present work was in progress. Thus, elastase shares the calcium binding properties of the two related serine proteases trypsin and chymotrypsin. Its affinity for calcium and gadolinium is even higher than that of these metals for the two other proteases (Tables I and II).

The calcium (gadolinium) ion-elastase complex is able to bind the substrate succinyltrialanine-*p*-nitroanilide and the inhibitor trifluoroacetyltrialanine with the same affinity as does the free enzyme. Moreover, the k_{cat} for the substrate is not altered by the presence of the two metals (Tables I and II). On the other hand, the elastolytic activity of elastase does not change in the presence of 1 mM calcium ion (Lewis et al., 1956). This is not true for trypsin, where calcium ions have been reported to activate the catalysis (Gomori, 1951). Furthermore, calcium decreases considerably the rate of trypsin autolysis (Nord and Bier, 1953), whereas it does not change the rate of elastase self-destruction.

The question now concerns the nature of the ion binding site of elastase. Our data show clearly that this site is located at a distance of 20 Å from the fluorine nuclei of the trifluoroacetylated inhibitor. We do not know precisely at which subsite the trifluoroacetyl group is bound. However, our previous studies have indicated that the trifluoroacetylated inhibitor cannot be bound at the S′ sites but is more likely bound to the S sites of the enzyme (Dimicoli et al., 1976). This view is in agreement with the x-ray investigations of Shotton et al. (1971) who have shown that trialanine is bound at S_{321}. The calcium binding site of elastase must therefore be located at a distance of about 20 Å from one of the S sites of the active center. The side chain of Asp-194, which has been identified as a part of the metal binding site of trypsin and chymotrypsin in solution (Abbott et al., 1975; Birnbaum et al., 1977), is located 7–11 Å from the S sites and can therefore be ruled out. More plausible candidates would be the side chains of Glu-70 and Glu-80 which are a part of the uranyl binding site in the crystal of elastase (Shotton and Watson, 1970). These residues are located at a distance of 14.5–19.5 Å from the S sites and are, therefore, likely to be involved in the binding of calcium and gadolinium. Experiments in progress using inhibitors which are fluorinated at both the C- and N-terminal parts of the peptide strengthen

this view, since both fluorine nuclei are located at a distance of about 20 Å from the gadolinium ion on the enzyme.

The dissociation constant of the elastase calcium ion complex has been measured at two different temperatures (Table I). The derived thermodynamic quantities are $\Delta H = 2.2$ kcal mol^{-1} and $\Delta S = 11$ eu at 4 °C and 14 eu at 35 °C. The positive entropy change is in accord with the electrostatic interaction proposed for the binding of calcium to elastase.

In addition to the ion binding properties of elastase described in this paper, the present investigation has led to the observation of new aspects to the interaction between elastase and trifluoroacetyltrialanine. These results will now be briefly discussed. The active center of elastase is composed of eight sites (Atlas, 1975). Inhibitors or substrates may, therefore, bind to the enzyme in different modes. The multiplicity of the binding modes obscures considerably the interpretation of the kinetic parameters K_m, k_{cat}, and K_I. The fact that the value of k_{off} for trifluoroacetyltrialanine does not depend upon the wide range of experimental conditions used to measure it suggests strongly that this inhibitor binds to elastase in a unique binding mode. Substrates or inhibitors structurally related to this compound should thus be useful tools for the further investigation of the active center of elastase.

As shown previously, trifluoroacetyltrialanine is a considerably more potent inhibitor that acetyltrialanine (Dimicoli et al., 1976). At first sight, one may hypothesize that this increase in affinity results from a strong interaction of the three fluorine atoms with the surface of the enzyme. Such a model, however, is contradicted by the similarities of the relaxation times in a diamagnetic complex ($T_{1EI} \simeq T_{2EI}$), which suggests that the fluorine atoms are still mobile within the tight enzyme inhibitor complex ($\tau_c \ll 10^{-9}$ s). The increased affinity is therefore more likely due to a fluorine-induced polarization of the adjacent carbonyl group of the inhibitor which facilitates the formation of a strong hydrogen bond between the inhibitor and the enzyme.

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